

Deeper Understanding of the Mechanism Promoting Gall Formation of *Zizania latifolia* under Fenaminosulf Treatment

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Abstract

Zizania latifolia is a popular aquatic vegetable in China because of its enlarged edible stems resulting from persistent infection by a fungal endophyte, *Ustilago esculenta*. Fenaminosulf (FM) is a common fungicide and microbicide. In *Z. latifolia* fields, appropriate spraying of FM not just controls diseases, but also promotes an earlier harvest of *Z. latifolia*. In this study, we show that the timing of gall formation was advanced and the plant's yield was increased significantly under a high concentration treatment of FM. Yet FM had a strong inhibitory effect on the growth of *U. esculenta* in vitro, while the transcript levels of some growth-related genes were all substantially downregulated. Through a transcriptome analysis, we found that FM directly affected the growth of *Z. latifolia* by altering the expression level of genes involved in plant-pathogen interactions, plant hormone signal transduction, and some metabolism pathways. By contrast, FM had little effect on *U. esculenta* growing inside of *Z. latifolia*. Taken together, these results provide a more in-depth understanding of the molecular processes that promote gall formation in *Z. latifolia*, while also identifying potential targets for genetic manipulation to improve the yield and quality of *Z. latifolia*, in a safer and more effective way.

Introduction

Zizania latifolia (Griseb.) Turcz. ex Stapf, belonging to the Oryzaceae of the Gramineae, is an asexual aquatic vegetable with an edible swelling structure which forms in the stem that has been cultivated in Asia for more than 2000 years¹⁻⁵. The harvested edible swelling stem of *Z. latifolia* are plump and tender, with little fiber, high protein, and abundant antioxidant properties that may prevent hypertension and cardiovascular disease⁶⁻⁸. According to photosensitivity and harvesting time of this species, more than 100 local varieties may be divided into two main ecotypes of *Z. latifolia*⁹. One ecotype is a single-season plant, harvested once per year in the fall (from August to November) after planting in spring. Another is a double-season crop, also planted in the spring, but harvested twice: in fall (hereon, 'Autumn-Jiao') and the following summer ('Summer-Jiao'). Temperature and illumination are the key factors influencing *Z. latifolia* growth.

The basidiomycetes fungus *Ustilago esculenta* could specifically colonize *Z. latifolia* hosts by invading their meristems. There, the mycelium of *U. esculenta* grows in or between the cells in the meristem near the stem tip, which prevents the infected plant from flowering and causes the formation of the localized, edible swelling structure¹⁰⁻¹⁴. The plant pathogen *U. esculenta* is unique among the smut fungi that are plant pathogens, which mainly parasitize grasses and cause 'smut disease'¹⁵⁻¹⁸, because it does not induce chlorosis symptoms in the host, or necrosis, but instead induces the formation of differentiated galls on the culm upon infection^{12, 19-23}. In China, *Z. latifolia* is commonly referred to as "*Jiaobai*" and categorized into three types according to the interaction mode with *U. esculenta*, normal *Jiaobai*, grey *Jiaobai*, and male *Jiaobai*^{24, 25}. The normal *Jiaobai* form edible galls at the base region of the plant and are full of fungal hyphae. The grey *Jiaoba* are filled with dark-colored teliospores that are often discarded due to their unacceptable taste and potential for triggering hypersensitivity pneumonitis when

consumed^{13,22,23}. The male *Jiaobai* without *U. esculenta* infection fails to produce any galls and will exhibit normal flowering^{1,20}. Some research has speculated that different phenotypes of normal and male *Jiaobai* in the fields arose from differentiated strains of *U. esculenta*, which spurred diverse host defense responses²⁵. In sum, *U. esculenta* infection is the critical factor for normal development of *Jiaobai*. The genetic interactions between plant and fungus were recently revealed by whole-genome sequencing and comparative transcriptome analyses²⁵⁻²⁹.

Fenaminosulf (FM, p-dimethylaminobenzenediazosodium sulfonate) is a common protective germicide, with special effects on the diseases caused by *Pythium* and *Aphanomyces* afflicting vegetable, cotton, tobacco, rice, and wheat crops³⁰⁻³². It is a very effective seed and soil treatment agent, able to control a variety of seed and soil borne diseases^{33,34}. FM is characterized by a strong absorption and penetration, such that it is readily absorbed by root and leaf surfaces. However, FM stimulates adverse effects when on human skin and it causes harm to the aquatic environment. Appropriate spraying of FM not only controls diseases in *Z. latifolia* fields but it also advances the harvest of the *Z. latifolia* crop. Currently, in some *Z. latifolia*-producing areas in China, when the harvest of edible swelling stem occurs may be regulated by spraying FM during the growth of *Z. latifolia*³⁵. Yet the lack of a scientific and rationally application method could cause FM to affect the growth and safety of *Z. latifolia* crops, run against the farmers' interests, and even pollute the environment. So far, the mechanism by which FM acts upon *Z. latifolia* and *U. esculenta* has not been elucidated in full. We investigated the effects of FM on the process of gall formation and carried out intensive research of molecular processes that could promote gall formation of *Z. latifolia*. The findings are beneficial for the further exploration and exploitation of gall formation effectors for improving the yield and quality of *Z. latifolia* in a safer and more efficient way.

In recent years, FM has been widely used for promoting the formation of the swollen stem in fields of *Z. latifolia*, but in the process its harvest timing has been significantly affected. In this report, except for the formation time of swollen stem, the yield and regulatory mechanism of *Z. latifolia* were monitored under different concentrations of applied FM. In parallel, the growth of *U. esculenta* under the action of FM was analyzed in the laboratory. In addition, transcriptome analysis was used to search for and elucidate FM's mechanistic action on *Z. latifolia* and *U. esculenta*. This study provides a comprehensive description of FM action on growth of *Z. latifolia* and *U. esculenta* and also provides a theoretical basis for the mechanism of swollen stem formation in *Z. latifolia*.

Results

FM affects the gall formation and yield of *Z. latifolia*

After treatment with different concentrations of FM, the timing of gall formation and yield of *Z. latifolia* was greatly affected. As Fig. 1A shows, the *Z. latifolia* began harvest from May 27 onward. Under the action of 3.6 g/L FM, the harvest time of *Z. latifolia* was markedly advanced. The peak harvest period of *Z. latifolia* under the 1.8, 0.9, and 0.45 g/L FM treatments was respectively delayed by 4, 17, and 19 days,

when compared with that under the 3.6 g/L FM application. The control group maintained a lower harvest volume during the entire harvest period.

The FM concentration applied not only affected the timing of gall formation, but also their total yield from *Z. latifolia* (Fig. 1B). When treated with a higher concentration of FM, a higher yield of *Z. latifolia* was harvestable. Under the 1.8 g/L and 3.6 g/L FM treatments, the yields of *Z. latifolia* respectively were 46.07 kg and 44.86 kg, which corresponded to 59.5% and 55.3% increases over the 0.9 g/L FM treatment (28.88 kg). Moreover, when compared with the yield of the 0.45 g/L treatment group (12.09 kg), their increments were 2.8-fold and 2.7-fold greater, respectively.

The harvest timing of *Z. latifolia* was the latest in the control group, for which it never reached the peak period during the whole harvest period (Fig. 1A, B). Accordingly, the control group had the lowest yield, at only 1.61 kg. Since fertilizer and water management and pest control were identical among the treatment groups, weather conditions were monitored (Fig. 1C, D). Around May 7, the *Z. latifolia* entered the period of gall formation, about 20 days before the onset of the harvest period (From May 7 to 27). According to the information provided by the local meteorological bureau, from May 7 to 12, the average temperature was around 20°C, a suitable temperature for gall formation. After May 17, however, the experimental field experienced high-temperature weather exceeding 30°C whose daily average temperature was above 25°C for 6 days. This spell of high temperature happened to occur in the critical period of gall formation in the control group.

FM affects the sporidia growth of *U. esculenta* in vitro

A high concentration of FM completely inhibited sporidia growth of *U. esculenta* in the YEPS culture. The sporidia began growing so long as the concentration was below 0.09 mg/ml; corresponding inhibition rates of 0.009 mg/ml, 0.018 mg/ml, 0.027 mg/ml, and 0.036 mg/ml FM on sporidia growth were 9.5%, 18.6%, 29%, and 37.8%, respectively (Fig. 2A). Hence, the inhibition rate increased with a rising concentration of FM. The FM resistance was quantified as EC₅₀ after exposure to FM irradiation. The EC₅₀ was 0.042 mg/ml.

Seventeen relative genes possibly involved in the altered responses of sporidia to FM (0.018 mg/ml) versus control group were assessed for their transcript levels via qRT-PCR with paired primers (Table S1). Sporidia isolated from teliospores could be divided into two strains: one containing *mfa1.2*, *mfa1.3*, and *pra1* alleles, while the other contained *mfa2.1*, *mfa2.3*, and *pra2* alleles, that were a mating-type alleles in *U. esculenta*. The expression levels of all of them were significantly repressed, by 38.1%–85.2% (Fig. 2B). The drastic repression of mating relative genes indicated that FM impaired the conjugation formation, the initial step of the mating process in *U. esculenta*.

Under the stress of FM (0.018 mg/ml), the expression levels of cell metabolism-related genes were also significantly repressed (by 21.8%–89.8%) in *U. esculenta* (Fig. 2C). Remarkably, *gpa3*, *kss1*, and *pkaC* of *U. esculenta* were all downregulated more than 80%. Among five chitin synthase genes (*chs1* through *chs5*) responding to FM (Fig. 2D), all were largely downregulated by 84.7%–89.2%. Transcriptional levels

of these genes related to cell metabolism and cell wall disturbing were all repressed in response to FM; this strongly implied FM could considerably affect the normal metabolic growth of *U. esculenta*.

Genome-wide expression analysis of FM effects upon *Z. latifolia* and *U. esculenta*

To determine how FM affects the gall formation in *Z. latifolia* and the interaction between it and *U. esculenta*, a transcriptome analysis was performed. The swollen stem galls harvest from the control group (without FM) and experimental group receiving the 3.6 g/L FM treatment were used to collect transcriptional information for *Z. latifolia* and *U. esculenta*. Samples with three replicates were prepared from these two groups.

Deep RNA sequencing produced the 9.2×10^7 and 8.6×10^7 valid reads for the *Z. latifolia*, 2.4×10^7 and 2.4×10^7 valid reads for the *U. esculenta* libraries, in the control and experimental group, respectively. These sequence reads were mapped onto the genome of *Z. latifolia* and *U. esculenta*, resulting in the identification of 46 092 and 7347 genes derived respectively from the *Z. latifolia* and *U. esculenta* libraries. Comparative analysis between the control and experimental groups' expression profiles revealed 663 and 912 transcripts upregulated and downregulated in *Z. latifolia*, and 34 and 24 transcripts upregulated and downregulated in *U. esculenta*, respectively (Fig. 3A).

GO enrichment analyses were used to annotate the function of differentially expressed genes (DEGs), which could be assigned to three major categories: molecular function (MF), biological process (BP), and cellular component (CC). In *Z. latifolia*, the unigenes were categorized into 45 GO terms, with most DEGs belonging to BP and MF (Fig. 3B). In the BP category, phosphorylation, protein modification, phosphorus- and phosphate-containing compound metabolic process, and macromolecule modification were the top four classes enriched by regulated transcripts. In the MF category, many genes were categorized as protein kinases and binding proteins. In *U. esculenta*, the number of DEGs was small (Table S3), and there was an average distribution in these three enriched GO categories.

As Fig. 3C shows, KEGG pathway enrichment analysis revealed a set of genes involved in galactose metabolism, butanoate metabolism, and starch and sucrose metabolism that were differentially expressed as enriched (corrected p-value < 0.2) in *U. esculenta*. In *Z. latifolia*, a fair number of DEGs (corrected p-value < 0.05) involved in plant–pathogen interaction, phenylpropanoid biosynthesis, zeatin biosynthesis, plant hormone signal transduction, flavonoid biosynthesis, biosynthesis of secondary metabolites, and biosynthesis of stilbenoid, diarylheptanoid, and gingerol were evidently enriched. Generally, those genes with a corrected p-value < 0.05 could be considered as an enriched item.

In *Z. latifolia*, the transcript levels of 77.7% of the DEGs (101 of 130) in the enrichment items were downregulated under the stress of FM (Table S2). Specifically, 94.4% (17 of 18), 73.7% (14 of 19), 83.3% (5 of 6), and 76.8% (43 of 56) of DEGs were downregulated dramatically in the process of plant–pathogen interaction (see also Fig. 4), phenylpropanoid biosynthesis, flavonoid biosynthesis, and biosynthesis of secondary metabolites. Notably, in the process of zeatin biosynthesis (7 genes) and stilbenoid, diarylheptanoid, and gingerol biosynthesis (4 genes), all of the DEGS were especially

downregulated. For the process of plant hormone signal transduction, 55% (11 of 20) of the involved DEGs were downregulated (Table S2; see also Fig. 5) and those were related to auxin signaling, seed dormancy, stress response, and disease resistance.

A previous study identified 170 and 205 putative host genes, along with 53 and 71 *U. esculenta* genes involved in the initial and late stage of culm gall formation²⁸. Compared with those genes, we found that 6 of 13 DEGs in *Z. latifolia* under FM stress had the same trend of change, in that they were all downregulated (Table 1). In *U. esculenta*, however, no DEGs with the same change pattern could be discerned by comparison in our experiment.

Discussion

The plant *Z. latifolia* forms a shuttle-like gall with a unique flavor, making it a widely cultivated aquatic vegetable in China. But under certain unfavorable conditions, such as high-dose radiation, water deficiency, unsuitable fungicide application, adverse growing temperatures, or if plants are infected with the T fungal strain (forming the grey *Jiaobai*) or escape from fungal infection (forming the male *Jiaobai*)^{22, 23, 38}, the yield of *Z. latifolia* may be substantially reduced.

As a common fungicide in *Z. latifolia* fields, FM is able to not only effectively inhibit disease but also promote the gall formation of *Z. latifolia*. Although the time of gall formation and yield of *Z. latifolia* in the control group were markedly affected by high temperature, in the experimental group they were significantly advanced and increased under the effect of FM and most pronounced under the high concentration treatment. The peak harvest period of *Z. latifolia* was 19 days sooner under the 3.6 g/L FM treatment than the 0.45 g/L FM one. When treated with 1.8 g/L and 3.6 g/L FM, the yields of *Z. latifolia* were respectively increased by 2.8-fold and 2.7-fold compared with the 0.45 g/L FM treatment. It seems reasonable to suggest that FM could significantly promote the gall formation of *Z. latifolia*, leading to an earlier harvest peak and its increased production.

The culm gall formation of *Z. latifolia* was affected not only by the external environment but also by the internal symbiotic smut. Previous studies had reported that *U. esculenta* gene expression contributes to culm gall formation in *Z. latifolia*^{28, 38}. Therefore, the use of fungicides in the field was done very cautiously to prevent potential negative impacts on the growth of *U. esculenta*, which would threaten the culm gall formation of its host, *Z. latifolia*. Here, the effect of FM on the growth of *U. esculenta* isolated from grey *Jiaobai* plants was tested in vitro. The sporidia of *U. esculenta* were incapable of growing under the action of high concentrations of FM, beginning to do so only when its concentration fell below 0.09 mg/ml. Clearly, the FM chemical compound has a strong inhibitory effect on *U. esculenta* growth. Under the stress of FM, the transcript levels of mating-type alleles, cell metabolism-related genes, and chitin synthase genes in *U. esculenta* were all repressed considerably. This suggests FM impaired the initial mating action and normal growth of *U. esculenta*, and interpretation that is in line with the effectiveness of FM as a fungicide.

Through the transcriptome analysis, we investigated changes in gene expression of the host *Z. latifolia* and the pathogen *U. esculenta* in response to FM, finding several noteworthy differences between control and stressed group. In *Z. latifolia*, functional analysis of DEGs revealed that most of them participate in phosphorylation, protein modification, macromolecule modification, phosphorus and phosphate-containing compound metabolic process, protein kinases, and binding proteins. In *U. esculenta*, the number of DEGs was small, and these genes were evenly distributed in three enriched GO terms categories.

The plant–pathogen interaction between *Z. latifolia* and *U. esculenta* is unique because *U. esculenta* induces the formation of differentiated galls upon infection of its host *Z. latifolia*¹². Unlike *Ustilago maydis*, which causes the production of large tumors on all aerial organs in maize plants¹⁷, *U. esculenta* induces culm gall formation in a more moderated way, by only inducing culm hypertrophy without obvious symptoms of tissue damage in other parts of its host^{22,23}. *Z. latifolia* responds strongly to its infection by *U. esculenta*, although the pathogen has the opportunity to survive in its plant tissues²⁸. Here we focused on changes of expressed genes in the processes of plant-pathogen interaction and plant hormone signal transduction by transcriptomic analysis, finding that FM elicited a plant response reaction to stimulus, thus implying a critical role of FM in promoting gall formations of *Z. latifolia*. Most of the identified DEGs exhibited decreased expression in the process of this plant–pathogen interaction. Compared with those genes involved in the swelling stage of culm gall formation, FM enhanced the function of the calcium-binding protein CML31 and disease resistance protein RPS2, which participate in plant response to external stimuli. Applying FM also influenced the expression of other genes, mainly those associated with inhibition, suggesting a stressed plant provides *U. esculenta* with superior opportunities to penetrate and expand in its plant tissues.

The induction of galls in host plants by microbes is undoubtedly dependent on the activity of plant hormones, particularly elevated levels of cytokinins and auxins, which trigger a signaling cascade leading to activation of mitotic cell division^{17,19,39}. In our study, the transcript levels of AUX1 (auxin influx carrier), TIR1 (transport inhibitor response 1-like protein), AUX/IAA (auxin-responsive protein IAA), and ARF (auxin response factor 23) were elevated, in contrast to GH3 (Indole-3-acetic acid-amido synthetase) and SAUR (auxin-induced protein) that were downregulated. This revealed that in a stressed plant, protein expression levels involved in auxin signaling prophase were all upregulated, and, upon reaching the prophase, the transcript levels of auxin signaling-related proteins underwent differing changes. This may arise from the need to maintain an adequate auxin balance in *Z. latifolia*. Furthermore, the expression levels of BSK and CYCD3, which are related to cell division and phytochrome-interacting factor TF known to promote stem growth and induce germination, were upregulated. The expression levels of other proteins involved in seed dormancy, stress response, and disease resistance were all downregulated. The expression level changes of these proteins were closely associated with gall formation occurring sooner in host plants. All the changed protein expression levels seem converged towards advancing the onset of gall formation of *Z. latifolia*.

Secondary metabolites, such as lignin synthesized through the phenylpropanoid biosynthesis pathway, can promote plant resistance to pathogenic infection⁴⁰. In our study, most of the DEGs in this pathway, as well as some enzyme genes in other metabolic pathways, showed diminished expression in the stressed plant group, suggesting these stressed plants provide a less resistant way for the penetration and expansion of *U. esculenta* in their plant tissues.

Under the stress of 3.6 g/L FM, the transcriptome of the pathogen *U. esculenta* in host *Z. latifolia* was also analyzed. The number of DEGs found was very small, however, at only 58 genes. There were 20 most enriched pathway terms in *U. esculenta* and these were mainly concentrated in metabolism pathways. By way of comparison with genes involved in culm gall formation, the DEGs of *U. esculenta* were not directly related to culm gall formation, revealing that FM did not promote culm gall formation of *Z. latifolia* by affecting the growth of *U. esculenta*, but rather by directly affect *Z. latifolia* plants.

Under field conditions, FM played a key role in promoting culm gall formation of *Z. latifolia*, leading to an earlier peak in harvesting and increased production. Although FM significantly inhibited the growth of *U. esculenta* isolated from *Z. latifolia*, it actually had little effect on *U. esculenta* when it grows within *Z. latifolia* hosts. From our investigation of genome-wide transcriptome profiles and expression profiles of *Z. latifolia*, we inferred that FM could directly affect the growth of *Z. latifolia* by altering gene expression level involved in plant-pathogen interactions, plant hormone signal transduction, and some metabolism pathway. Still, besides their possible association with gall formation under FM stress, how the candidate genes function in terms of inducing gall formation remains unknown. The effects of FM on *Z. latifolia* could bring huge economic benefits to farmers, but the safety of its use and the residue it leaves must be seriously considered. Some studies reported that FM could induce a wide range of damage to DNA in *Allium. cepa* root nuclei³² and it was slightly toxic to *Lepomis macrochirus* and *Oncorhynchus mykiss*^{41, 42}. Although the concentration of FM used in our field experiment was relatively low, and FM easily dissolves under sunlight, we recommend that is still should be used carefully. This will spur us to find a safer way to promote gall formation of *Z. latifolia*. Collectively, the findings in this study deepen our understanding of molecular processes underlying the promotion of gall formation in *Z. latifolia*. They could prove timely and useful in further exploratory research and for exploiting the gall formation genes to improve the yield and quality of *Z. latifoliaa* in way that is both safer and more efficient.

Conclusions

We conclude that FM has a positive, promoting effect on culm gall formation of *Z. latifolia*, advancing the peak time of its harvest and increasing its yield under field conditions. Although *U. esculenta* is essential for culm gall formation, the pathogen is little affected by FM in the process of promoting culm gall formation, which means that FM does not impair culm gall formation through the growth of *U. esculenta*. Our results imply that FM could directly affect the growth of *Z. latifolia* by altering the expression levels of key genes involved in plant-pathogen interactions, plant hormone signal transduction, and some metabolism pathways. The effects of FM on *Z. latifolia* will bring huge economic

benefits, but due to the safety issues and residue of this chemical compound's application, we strongly recommend using FM with extreme caution.

Materials And Methods

I genes (Table S1) in the RNA extracts of sporidia YEPS cultures during the 10-day incubation at 28°C were determined via qRT-PCR. All total RNAs were extracted from the stressed (0.018 mg/ml FM) and unstressed cultures (without FM) using RNAiso Plus (Takara, Japan), from which 5-ug RNA samples were transcribed using a PrimeScriptTM RT reagent kit (Takara, Japan). A dilution (10 ng/μl) of the synthesized cDNA was then used as a template for qRT-PCR with paired primers (Supplementary Table 1) for each gene. The fungal 18S rRNA served as internal standard. Each qRT-PCR assay included three samples as replicates. The relative transcript level of a given gene was calculated as the ratio of its transcript in the stressed culture over that in the unstressed control³⁶.

RNA extraction, cDNA library construction, and RNA-Seq

Jiaobai samples were selected from the control group and the 0.6 g/L FM group in the experimental field plots. The total RNA of *Z. latifolia*, and that of *U. esculenta*, were extracted from the culms, by the TRIzol Reagent (Invitrogen, USA) and following the manufacturer's instructions. All samples were treated with DNase I (New England Biolabs, MA, USA). The purity and quantity of total RNA was respectively assessed with a NanoPhotometer[®] spectrophotometer (IMPLEN, CA, USA) and a Qubit[®] 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was confirmed using the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The total high-quality RNA isolated from three independent biological replicates of *Jiaobai* samples were used individually to construct cDNA libraries.

The RNA-Seq of two samples, namely control group and 3.6 g/L FM stressed group, each with three biological replicate, was performed on the Illumina HiSeq 2000 platform from which 150-bp paired-end reads were generated. Raw sequences were deposited in the NCBI Short Read Archive (SRA) database (<http://www.ncbi.nlm.nih.gov/Traces/sra/>). The accession number of RNA-seq data was PRJNA 669494 for *Z. latifolia* and PRJNA 669466 for *U. esculenta*. Raw reads in the FASTQ format were first filtered, by removing any reads containing adapter sequences and the low-quality reads. At the same time, the Q20, Q30, GC-content, and sequence duplication level of the clean data were each calculated. Cleaned reads containing both host and *U. esculenta* sequences were mapped onto the *Z. latifolia* reference genome²⁷ and *U. esculenta* reference genome²⁹, using the Bowtie2 software tool³⁷.

Transcriptome annotation, expression profiling, and data analysis

Gene function was annotated by homology searches against the NCBI non-redundant protein (Nr) database, NCBI nucleotide (Nt) database, Swiss-Prot protein database, euKaryotic Orthologous Groups (KOG), Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), and the Protein family (Pfam) database.

Expression levels of genes were measured using counts of reads normalized by their respective lengths, in the Cufflinks 2.0.2 package under its default settings (<http://cole-trap-nell-lab.github.io/cufflinks/>) for normalization (genometric), followed by their distribution analysis in terms of Fragments Per Kilobase of exon per Million mapped reads (FPKM) units⁴³. The DEGseq package was used to identify those DEGs between control group and 3.6 g/L FM stressed group; it provides statistical routines for determining differential expression levels in digital gene expression data by applying a model based on the negative binomial distribution. Resulting p-values were adjusted with the Benjamini and Hochberg's approach for controlling the false discovery rate (FDR). Genes with an adjusted p-value < 0.05 as detected by DESeq were designated as differentially expressed. The fold-changes for a given gene's expression between samples were calculated as log₂ (treatment FPKM value/control FPKM value).

Declarations

Data Availability

The data will be available online.

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Author contributions

Jianming Chen conceived the study; Fang Li performed the study; Juefeng Zhang and Haiying Zhong participated in some experiments. Fang Li and Jianming Chen analyzed the data and wrote the paper.

Additional Information

Competing Interest: The authors declare no conflict of interest.

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Tables

Table 1. Gene expression results for the full list of overlapping transcripts between paired libraries, comparing (i) differentially expressed genes (DEGs) in fenaminosulf -stressed *Zizania latifolia*, and (ii) DEGs involved in the initial triggering and swelling stage of culm gall formation in *Z. latifolia*.

Locus tag	Annotation	log2[foldchange] (i)	log2[foldchange] (ii)
Zlat_10004561	Calcium-binding protein CML31	-1.5199	-14.0995
Zlat_10006578	Disease resistance protein 2 RPS2	-1.8883	-10.2046
Zlat_10001910	Peroxidase 52 PER52	2.1065	-8.611
Zlat_10008844	Cationic peroxidase SPC4	-1.4769	3.4341
Zlat_10001161	Cytokinin dehydrogenase 4 CKX4	-1.9672	-9.4798
Zlat_10003321	Adenylate isopentenyltransferase 1 IPT1	-1.6601	1.2887
Zlat_10026402	Gibberellin 2-beta-dioxygenase 8	-2.081	-9.0526
Zlat_10019656	4-hydroxyphenylacetaldehyde oxime monooxygenase	-2.4564	-10.676
Zlat_10020835	1-aminocyclopropane-1-carboxylate oxidase	-2.4359	-7.6176
Zlat_10006128	Isocitrate lyase	-100	6.7474

Supplementary Data

Additional Supplementary Material may be found in the online version of this article.

Table S1. Paired primers used for qRT-PCR in *Ustilago esculenta*.

Table S2. List of differentially expressed genes in *Zizania latifolia* under FM stress.

Table S3. List of differentially expressed genes (DEGs) in *Ustilago esculenta* under FM stress.

Figures

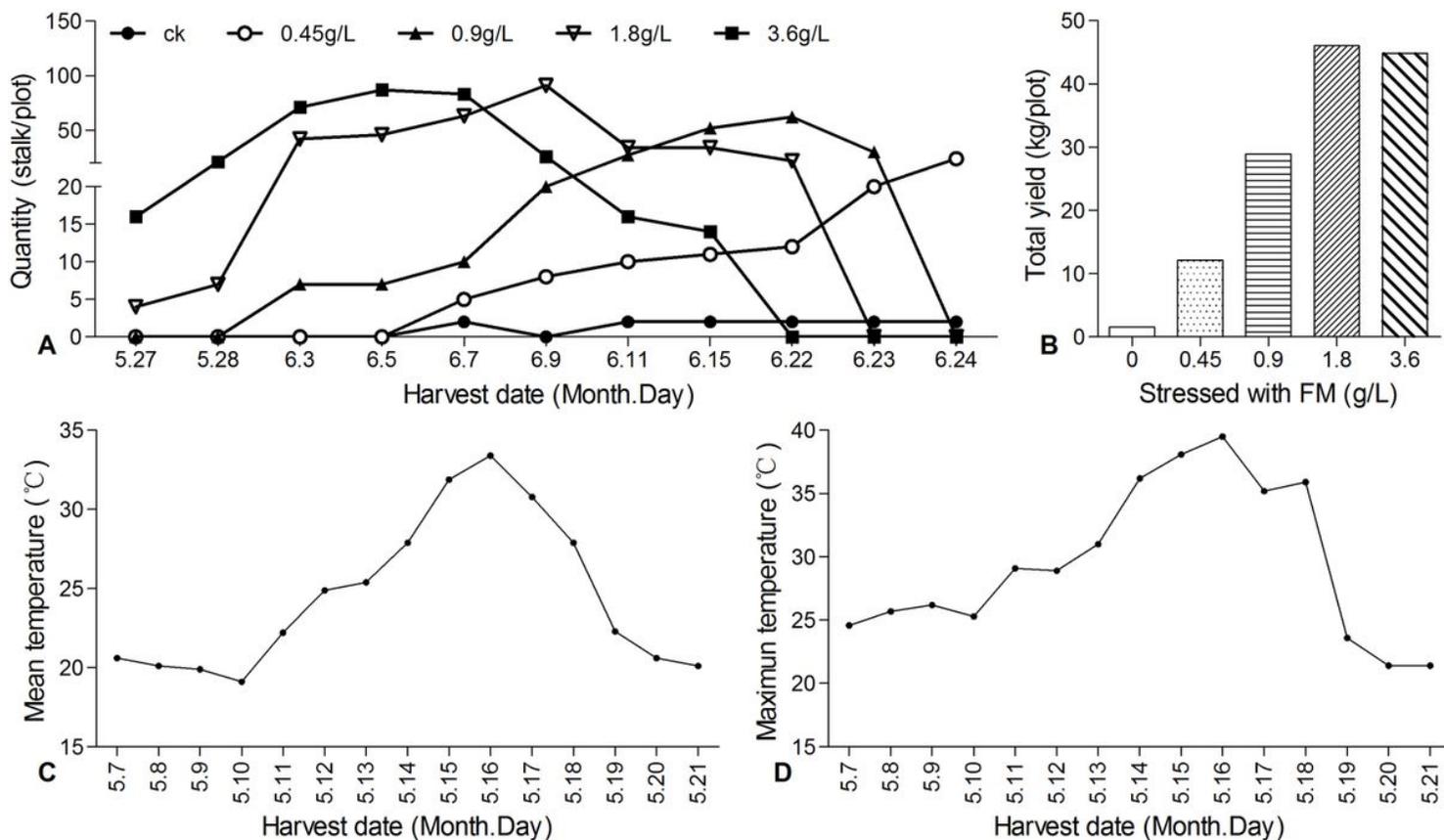


Figure 1

Effects of fenaminosulf (FM) on the gall formation and yield of *Zizania latifolia*. (A) Daily yield of *Z. latifolia* recorded after incubation in the presence of different concentrations of FM. The stems were collected from May 27 to June 24 from each plot treated with 0, 0.45, 0.9, 1.8, and 3.6 g/L FM. (B) The total yield of *Z. latifolia* under different concentrations of FM. The total weight of *Z. latifolia* in each plot treated with 0, 0.45, 0.9, 1.8, and 3.6 g/L FM was calculated. (C) and (D). Mean and maximum temperatures during the gall formation period in the field experiment. The mean and maximum temperatures of each day during the harvest period from May 7 to 21 were obtained from local meteorological bureau.

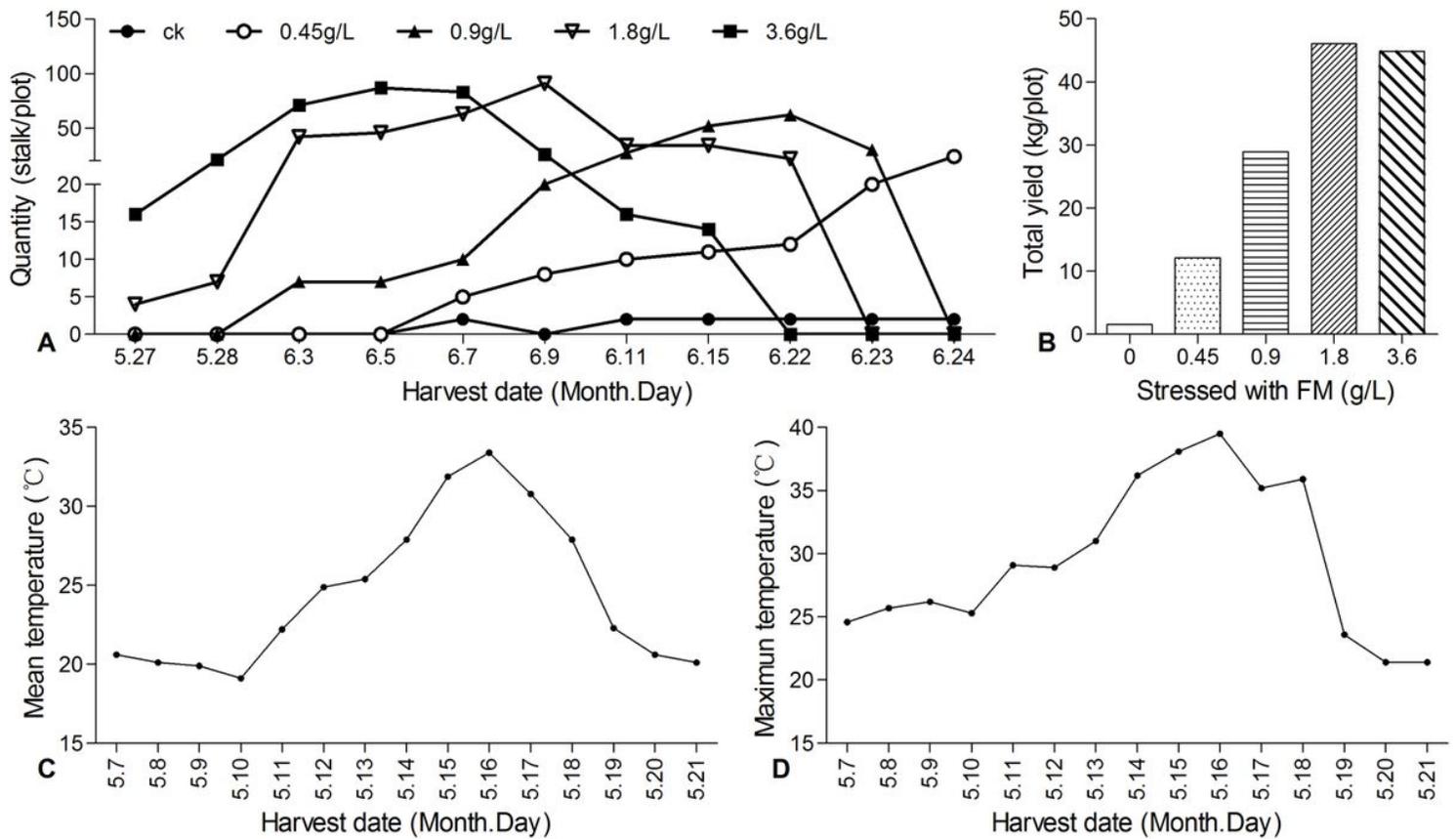


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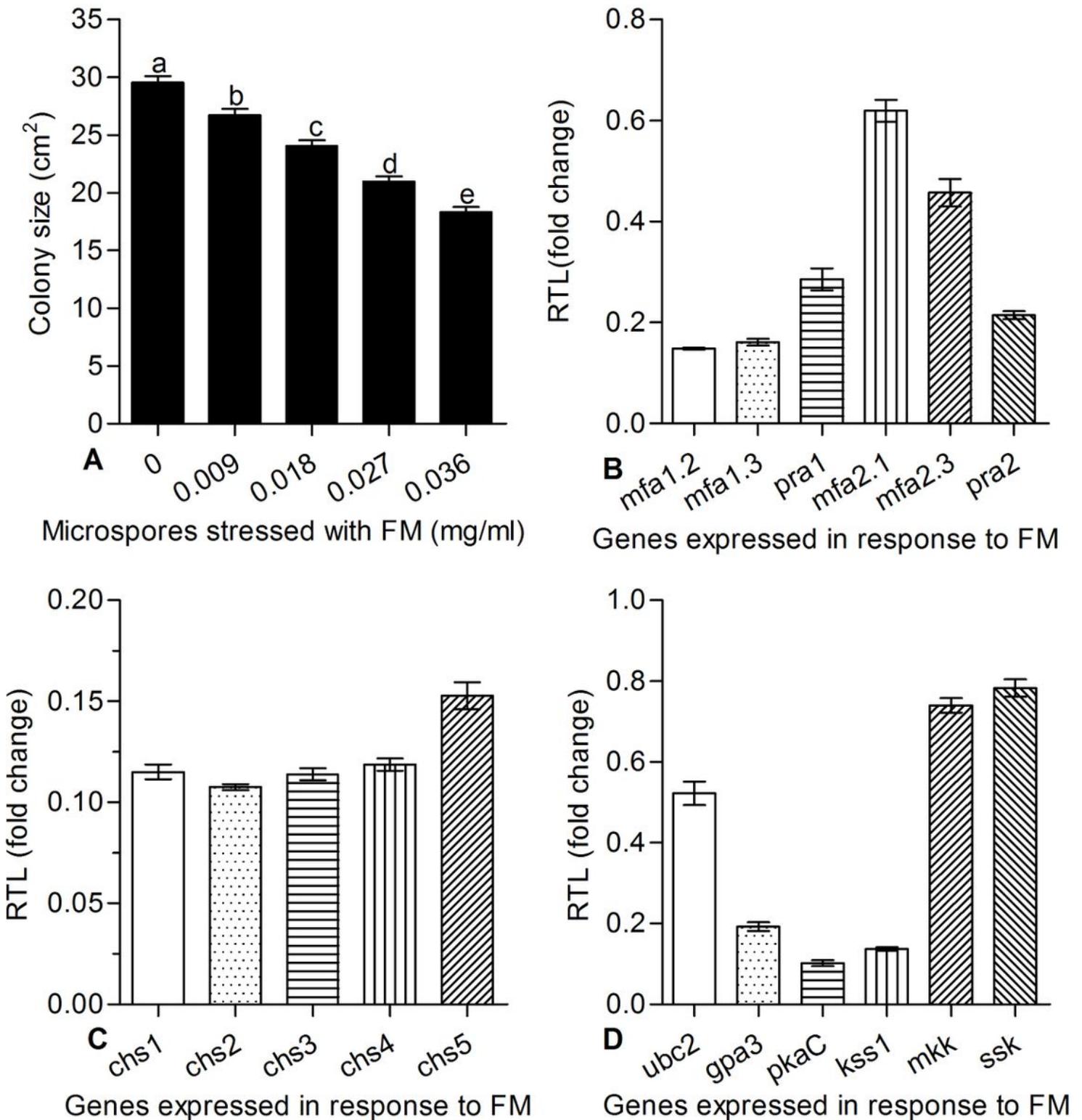


Figure 2

Effects of fenaminosulf (FM) on the growth of *Ustilago esculenta*. (A) Growth of *U. esculenta* on stressed media. The sporidia of *U. esculenta* isolated from grey Jiaobai were grown on PDA with gradient concentrations of FM (0.45, 0.9, 1.8, and 3.6 mg/ml). Colony sizes were calculated after 10-day growth at 28°C. Different letters above the column bars indicate significant differences ($p < 0.05$). Error bars: SD of the mean from three replicate assays. (B–D). Relative transcript levels of selected stress-responsive

genes in the stressed cultures of *U. esculenta* (versus the control). The cultures were stressed with 0.018 mg/ml FM during a 10-day incubation at 28°C and assayed via qRT-PCR with paired primers in the Table S1. Error bars: SD of the mean from three cDNA samples.

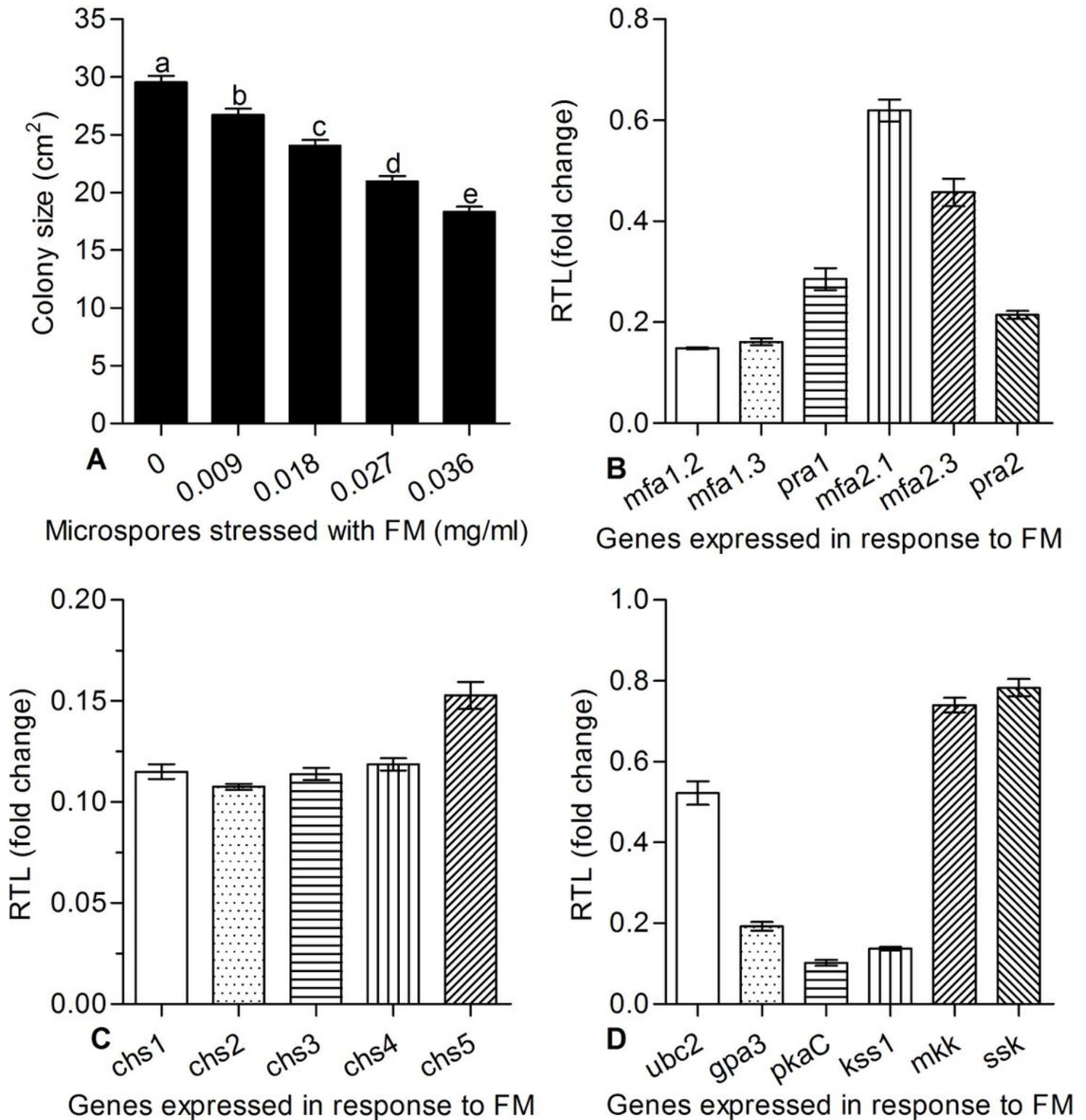


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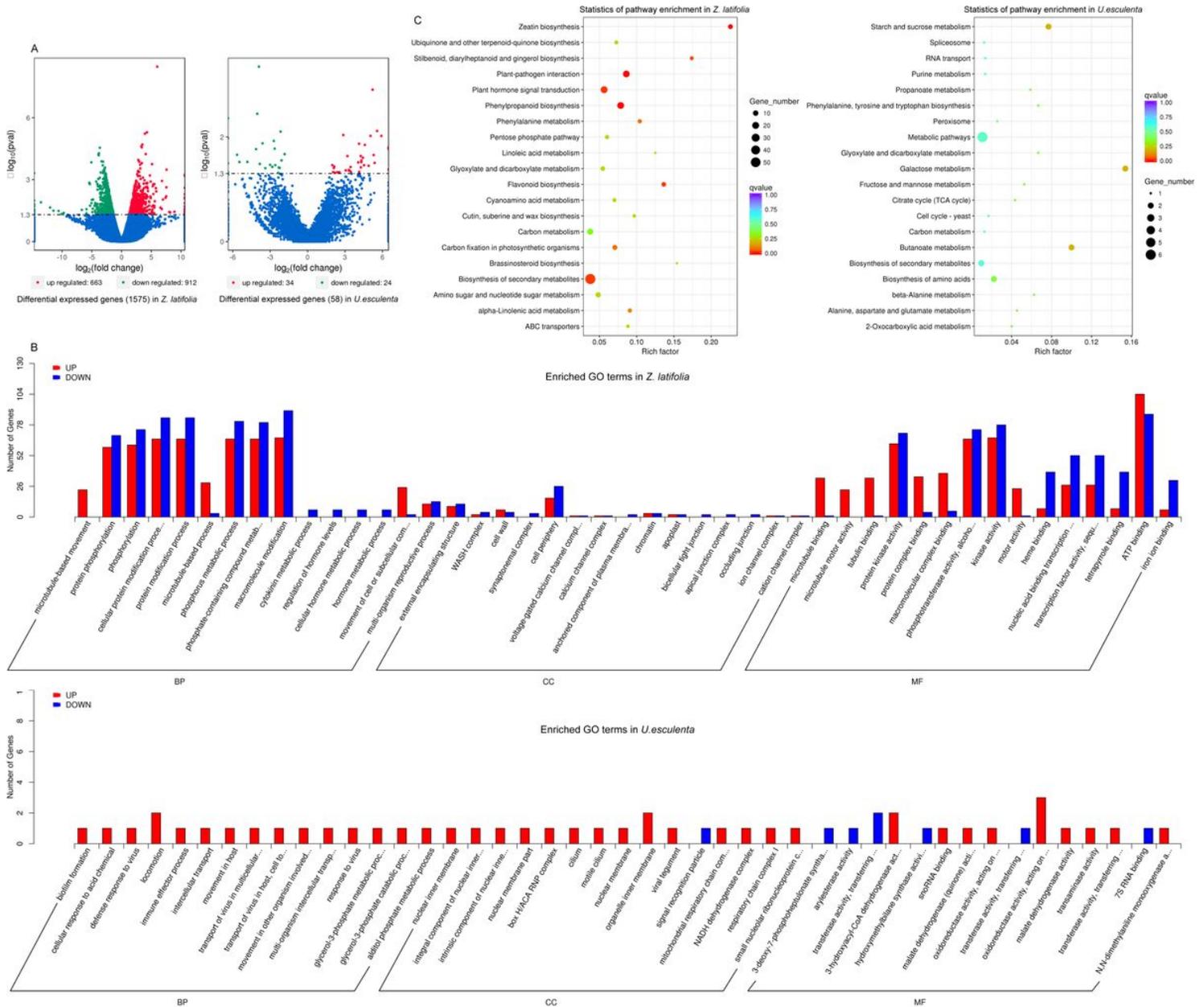


Figure 3

Overview of RNA-Seq data. (A) Volcano-plot of differentially expressed genes (DEGs) from the stressed group compared with the control in *Zizania latifolia* and *Ustilago esculenta*, respectively. Blue- and red-colored splashes represent significantly upregulated and downregulated genes. Blue splashes represent genes without significant differences in their expression levels. (B) Gene ontology (GO) classification of DEGs between stressed culms and the control in *Z. latifolia* and *U. esculenta*, respectively. Along the

vertical axis is the enriched GO term, and on the horizontal axis is the number of DEGs in a given term. Red- and blue-colored bars represent upregulated and downregulated genes, respectively. (C) Kyoto Encyclopedia of genes and Genomes (KEGG) pathway enrichment analysis of DEGs in *Z. latifolia* and *U. esculenta*, respectively. The ordinate represents the pathway name, the abscissa represents the rich factor, and the point size represents the number of DEGs in that pathway, while the point color denotes the differing Q-value ranges.

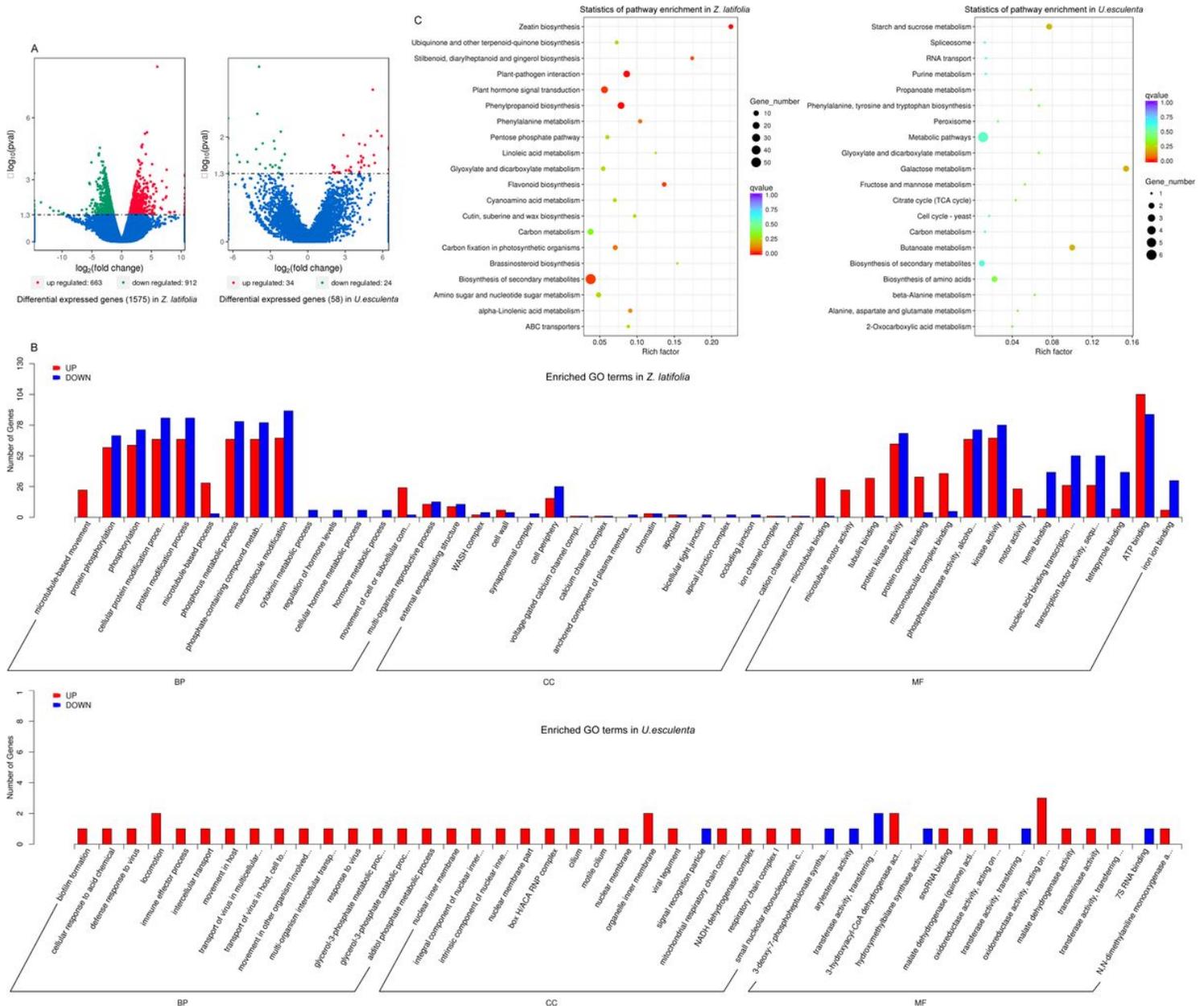


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PLANT HORMONE SIGNAL TRANSDUCTION

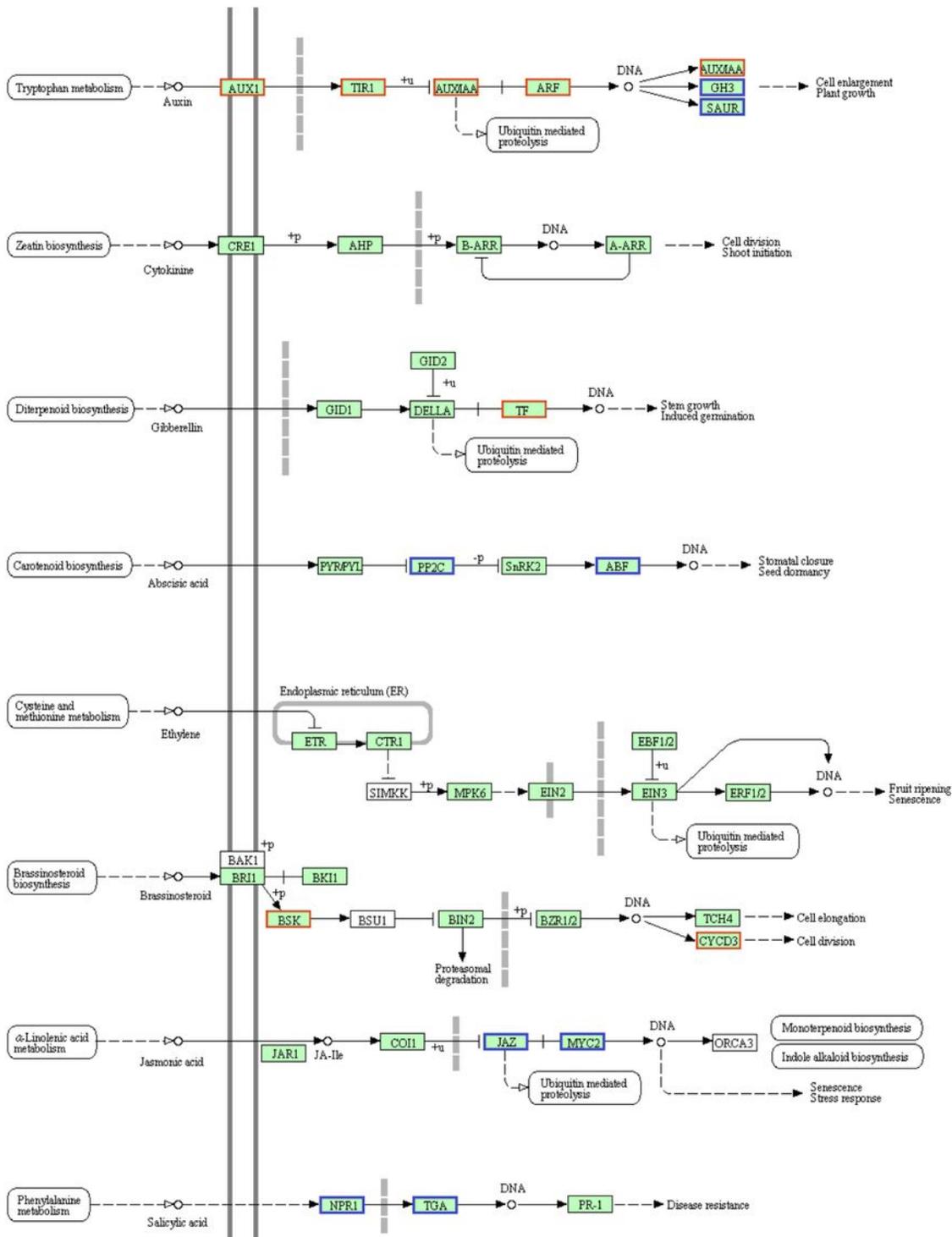


Figure 4

Gene expression effects of fenaminosulf on plant-pathogen interactions in *Zizania latifolia*. Genes downregulated (boxed in blue) or upregulated (boxed in red) in the stressed culm group versus control.

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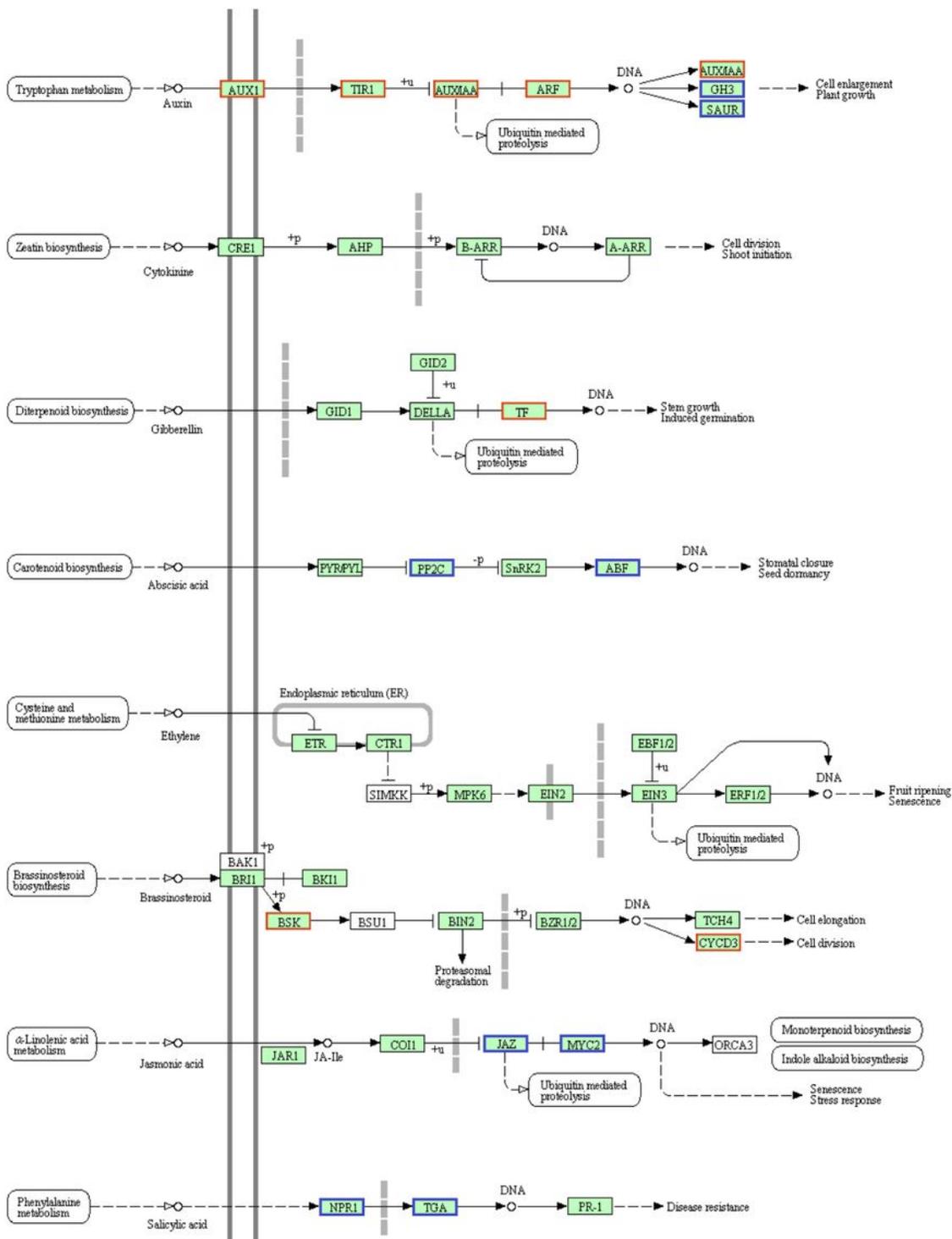


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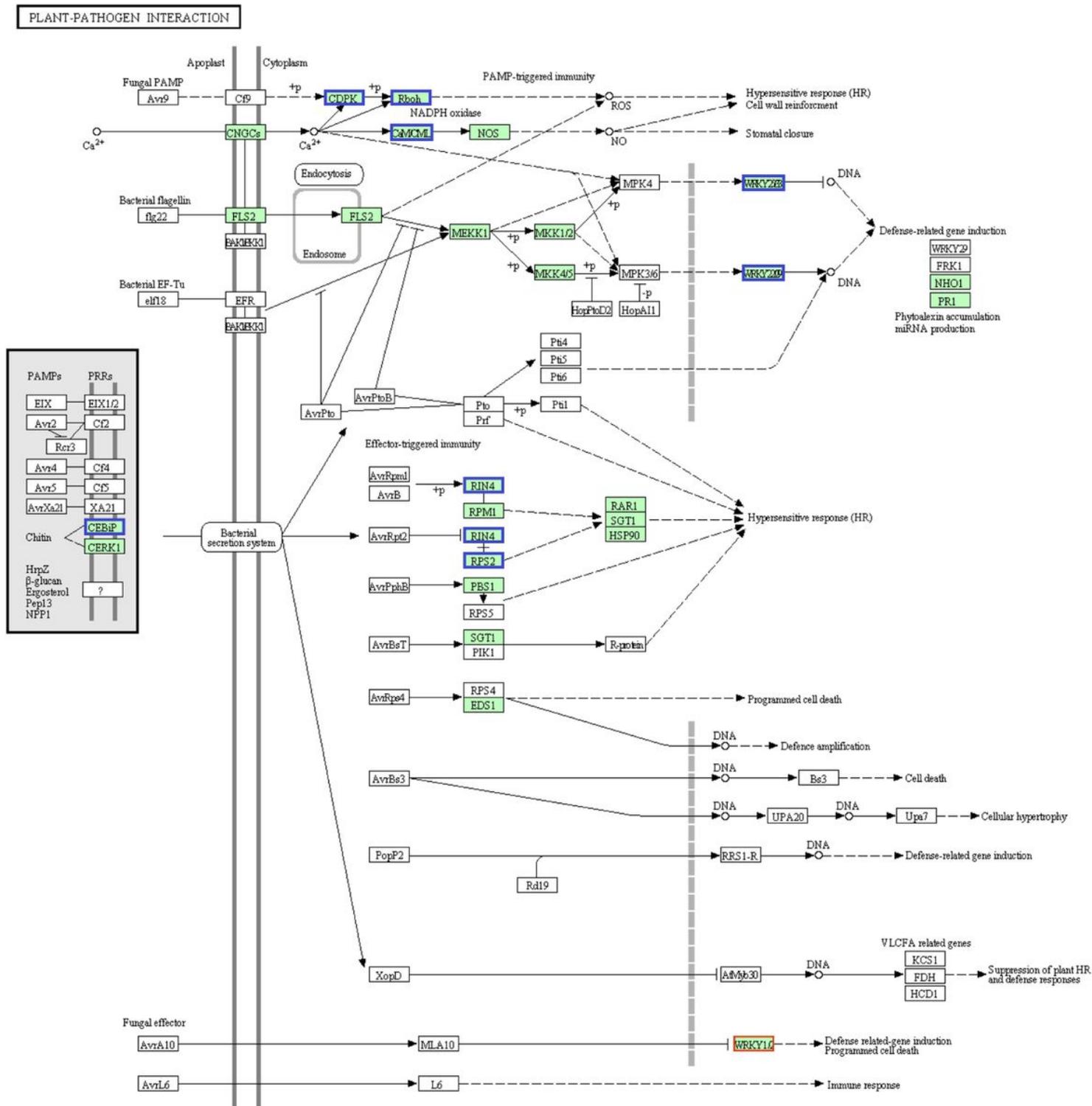


Figure 5

Gene expression effects of fenaminosulf on plant hormone signal transduction in *Zizania latifolia*. Genes downregulated (boxed in blue) or upregulated (boxed in red) in the stressed culm group versus the control.

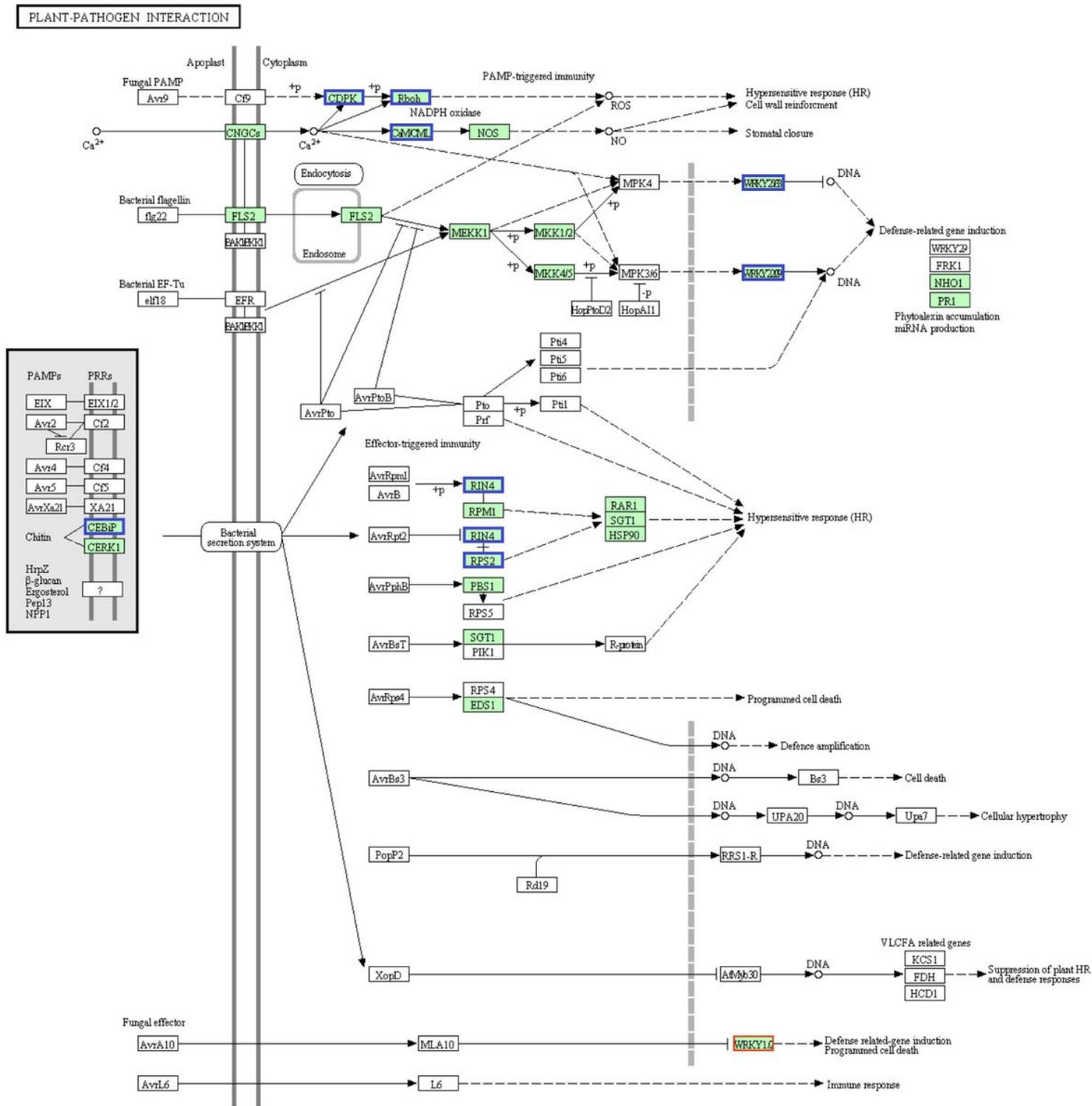


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